NISTIR 4529

Alaska Marine Mammal Tissue Archival Project:

Revised Collection Protocol

Paul R. Becker

U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Ocean Service
Office of Ocean Resources Conservation and Assessment
Arctic Environmental Assessment Center
Anchorage, Alaska 99513

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Alaska Department of Fish and Game
Alaska and Inuvialuit Beluga Whale Committee
Alaska Eskimo Whaling Commission
Aleut Community (St. Paul)
English Bay Village Council
Eskimo Walrus Commission
Kawerak, Inc.
National Marine Fisheries Service
New Chenega Bay Village Council
Nome Eskimo Community
North Slope Borough Department of Wildlife Management
North Slope Borough Fish and Game Management Committee
Norton Sound Health Corporation
Point Hope Whalers Association
Point Lay Village
Sitnasuak Native Corporation
Tanadgusix (TDX) Corporation
Tatitlek Village Council
U.S. Fish and Wildlife Service

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Disclaimer

Certain commercial equipment, instruments, or materials are identified in this Report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Contents

Figures

Tables

INTRODUCTION

In 1987, the Outer Continental Shelf Studies Program of the Minerals Management Service (MMS) provided funds to the Ocean Assessments Division (OAD) Alaska Office[1], National Oceanic and Atmospheric Administration (NOAA), to establish and conduct a program of collecting tissues from Alaska marine mammals and storing them under conditions which would allow for future analyses for substances indicative of contamination from offshore oil and gas, and mining activities. It was believed that such a collection of samples could provide a valuable source of information to help evaluate effects associated with the development of Alaska's coastal areas and with any future oil spills occurring in Alaska's coastal waters.

In response to this need to establish an "archive" of marine mammal tissues, the OAD Alaska Office entered into a cooperative effort with the National Institute of Standards and Technology (NIST) Center for Analytical Chemistry to plan, develop, and maintain such a program. A key factor in establishing this relationship was NIST's extensive experience in the development of detailed and rigorous tissue sampling procedures and its sophisticated facilities for the cryogenic storage of these samples. Through the work of NOAA/NIST, the *Alaska Marine Mammal Tissue Archival Project (AMMTAP)* was established in 1987.

The collection, packaging, and shipment of samples are being conducted by NOAA and NIST through cooperative efforts with other management, survey, and research programs of agencies such as the National Marine Fisheries Service (NMFS), the Alaska Department of Fish and Game (ADF&G), the North Slope Borough Department of Wildlife Management (NSB DWM), and the U.S. Fish and Wildlife Service (USFWS).

Cataloging and archiving of samples are conducted at the *Alaska Marine Mammal Tissue Archive*, which is maintained by NIST in its National Biomonitoring Specimen Bank (NBSB), Gaithersburg, Maryland. The NBSB, which is designed for long-term cryogenic storage of environmental samples, is the result of 10 years of development involving cooperative efforts between NIST and EPA, and several years of comparative studies with specimen archiving programs in Europe and Canada (Wise and Zeisler, 1984; Wise *et al.*, 1989). Procedures used in the *Alaska Marine Mammal Tissue Archive* are consistent with those employed by the NBSB in support of NOAA's *National Status and Trends Program* (Lauenstein, 1986).

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[1]*In 1990, the OAD Alaska Office became the Arctic Environmental Assessment Center (AEAC) of the Office of Ocean Resources Conservation and Assessment (ORCA), NOAA.*

The collection protocol used by the *AMMTAP* was originally published in a project description document in 1988 (Becker *et al.*, 1988). Since then, additional field experience with several species in various geographical areas of Alaska has resulted in some refinements of the original procedures. This report presents the most up-to-date collection protocol used by the *Project*.

Goal and Objectives

The goal of the *Alaska Marine Mammal Tissue Archival Project (AMMTAP)* is to archive a representative collection of Alaska marine mammal tissues for future analyses and documentation of long-term trends in environmental quality. The *AMMTAP* emphasizes the use of standardized rigorous sampling and archival protocols, which includes establishing the best conditions for maintaining sample integrity during storage for relatively long periods of time.

The objectives of the *AMMTAP* are:

1. Collect Alaska marine mammal tissues that are suitable for determining levels of organic and inorganic toxic substances.

   Collections of tissues for the archive are being limited to freshly killed animals taken by researchers or taken in subsistence hunts. When a sample archived by this project is analyzed, the researcher must have confidence that the sample was collected as prescribed in acceptable protocols (refer to *Protocol*, pages 9-28). No stranded dead animals nor old specimens archived from past programs are accepted by this project.

2. Transport, catalog, and curate the tissues in a condition suitable for long-term storage and eventual analyses.

   After collection, samples are packaged, transported, cataloged, and archived according to protocols consistent with those employed by the National Biomonitoring Specimen Bank. Storage is under liquid nitrogen vapor at -150°C, which is the best condition available for minimizing sample degradation. Samples will be selected by NOAA/MMS for future chemical analysis. Emphasis will be on those substances associated with offshore mineral extraction. Requests for archived samples by other researchers and agencies will be considered on a case-by-case basis (refer to *Sample Access Policy*, page 8).

3. Determine the most appropriate collection protocols for long-term specimen banking of marine mammal tissues.

The original field collection protocols (Becker *et al.*, 1988) were based on a 1987 pilot study of northern fur seal sampling. These protocols continue to be evaluated as to their practicality and suitability for obtaining uncontaminated samples as more species are sampled from different geographical areas and under different conditions in Alaska.

BACKGROUND

In the past decade, the concept of archiving biological and environmental samples for retrospective analysis has been recognized by the international scientific community as an important component of systematic environmental monitoring. Specimen archival banks have been established in the United States, Canada, and Europe. The long-term storage of carefully selected, representative samples in such banks is an important complement to the real-time monitoring of the environment. These archived samples permit:

1. the use of new and innovative analytical technology that was not available at the time the samples were collected, for clear state-of-the-art identification and quantification of analytes of interest, and

2. the identification and quantification of analytes that are of interest at the present, but that were not of interest at the time the samples were collected.

The retrospective analysis of archived samples allows the comparison of present and past analytical techniques and values, thus providing continued credibility of past analytical values, and allowing flexibility in environmental monitoring programs.

Marine mammals are long-lived and are generally considered as top predators in the marine environment. Chemical analysis of their tissues can be particularly useful in determining whether bioaccumulation of toxicants (and potential biological effects) associated with human industrial activities, including offshore petroleum and mineral extraction, is occurring in marine food chains. The collection of marine mammal tissues over a period of several years will provide an archive of samples that can be used to determine baseline contaminant levels against which future contaminant measures can be compared.

Selection of Tissues and Species for Sampling

The tissues originally selected for routine sampling were blubber, liver, kidney, and muscle. The selection of these four tissues/organs were based on a set of criteria described in Becker *et al.* (1988):

- A minimum of two 150 g samples can be obtained from the tissue.
- The tissue will provide a homogeneous sample.

- The sample is conducive to precise anatomical description.
- The tissue is accessible to sampling techniques.
- The tissue has the potential for concentrating both inorganic and organic substances.

The primary tissues chosen for sampling were blubber and liver. Blubber, due to its high lipid content, concentrates organic toxicants to relatively high levels, therefore, justifying its selection for sampling. The liver, which is a major detoxification site for xenobiotics, is suitable for measuring all known environmental toxicants plus biotoxins. The liver generally has sufficient lipid content that it is suitable as an accumulator of organic as well inorganic substances and may also represent a higher proportion of metabolites than other tissues. Because of the tendency for several of the toxic metals (particularly Cd) to concentrate to relatively high levels in the kidney, this organ was also selected to be one routinely collected by the *AMMTAP*.

After the first year of sampling, muscle was deleted from the list of tissues routinely archived by the *Project*. This was due to the relatively low levels of toxicants usually associated with muscle as compared to the other three tissue types, the difficulty in obtaining a uniform sample uncontaminated by intermuscular fat and connective tissue, as well as the difficulty in arriving at homogeneous analytical aliquots during cryogenic homogenization of the muscle samples.

In addition to the three tissues collected for cryogenic storage, other samples that can aid in interpreting the results of chemical analyses of these principal tissues are routinely collected from the animals (Table 1).

Twelve species of marine mammals were originally selected for sampling (Becker *et al.*, 1988). Those species of subsistence value to Alaskans, particularly in the Arctic and Bering Sea, have been emphasized in the actual collections. Samples have been collected from ringed seals (*Phoca hispida*), harbor seals (*P. vitulina*), bearded seals (*Erignathus barbatus*), northern fur seals (*Callorhinus ursinus*), Steller sea lions (*Eumatopias jubatus*), and belukha whales (*Delphinapterus leucas*). In all cases the source of materials were freshly killed animals taken in native subsistence hunts. Other species of subsistence value which the *Project* has yet to sample are: bowhead whale (*Balaena mysticetus*), Pacific walrus (*Odobenus rosmarus divergens*), polar bear (*Ursus maritimus*), and spotted seal (*P. largha*).

Table 1. Samples routinely collected by the *AMMTAP*.

LN2-Frozen Samples (shipped to the NBSB):
Blubber
Liver
Kidney
*Muscle
Ancillary Samples:
Liver, histological
Kidney, histological
Teeth and claws for age determination
Bile (LN_2-frozen) for PAH metabolite screening
Stomach contents for food identification

**No longer routinely collected*

Coordination Requirements

From the beginning, the work of the *AMMTAP* has required extensive coordination with many different organizations and individuals, both inside and outside Alaska (Figure 1). The Alaska native communities have a keen interest in the health and well-being of marine mammals, since they form an important subsistence resource for these communities; therefore, the *Project* has placed particular emphasis on establishing and maintaining a close working relationship with Alaska Native organizations.

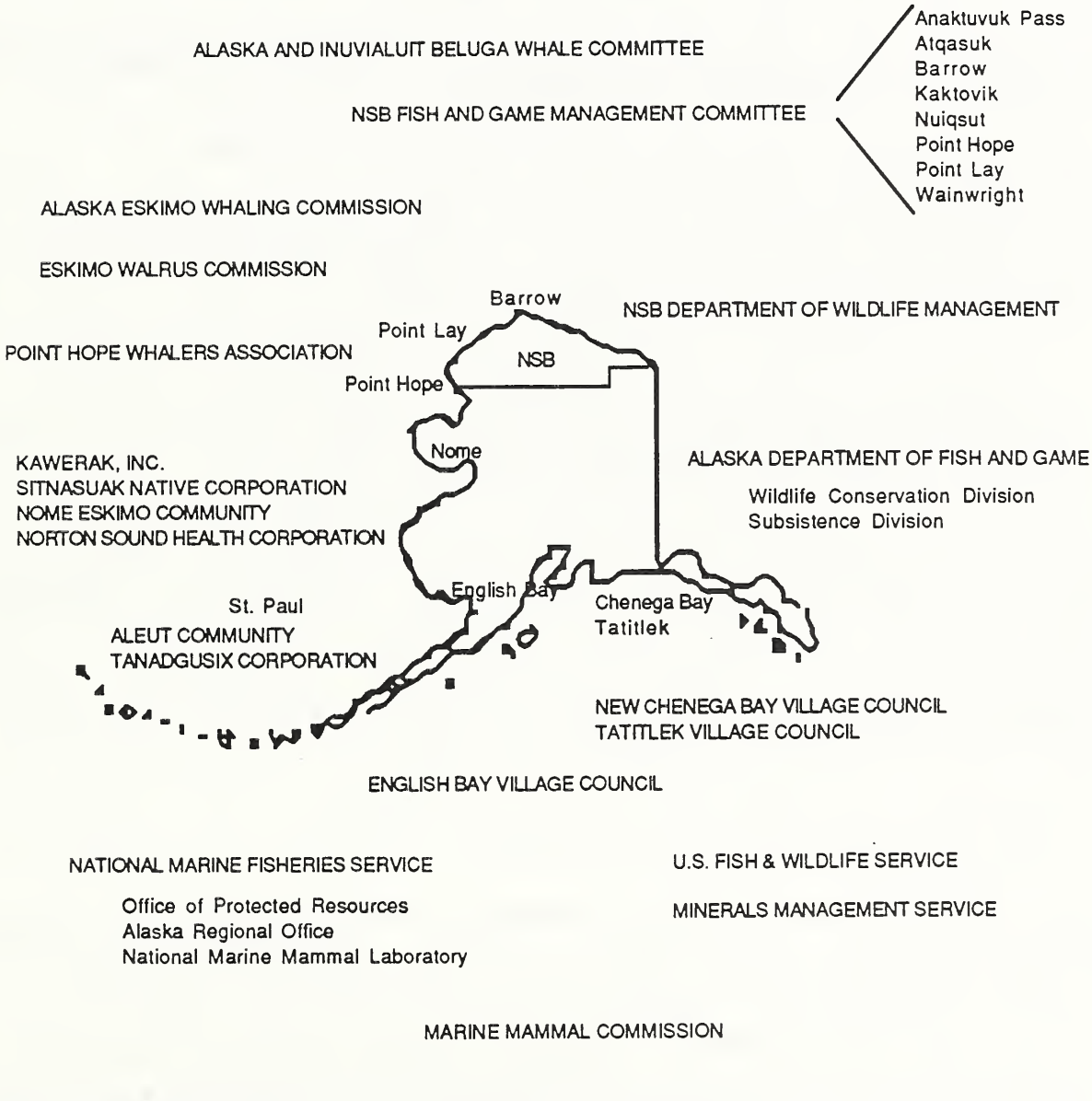

Figure 1. Organizations with which the *AMMTAP* has coordinated during its planning and operations (NSB = North Slope Borough).

SAMPLE ACCESS POLICY

Requests for any tissues for analyses or other uses will be considered. However, release of the tissues to investigators not associated with the *AMMTAP* is contingent upon the approval of MMS, after consultation with AEAC/NOAA and NIST. Release of these tissues will depend on a determination that a sufficient amount of requested tissues exists beyond anticipated sampling or analytical needs of the *AMMTAP*, that the proposed work can only be practically satisfied through use of archived samples, and that such analyses or uses will be performed cooperatively by the requesting organization and the *AMMTAP*.

A request for samples requires a written proposal that includes the following information:

1. Individual/organization making the request.
2. Purpose of the proposed study.
3. Researcher/laboratory conducting the analyses.
4. Kind of analyses to be performed/purpose of analyses.
5. Procedure/instrumentation/detection level.
6. Accuracy/precision to be expected.
7. Quality control procedures to be used.
8. A justification for the need to use archived samples rather than freshly collected tissues.
9. Agreement to provide AEAC/NOAA, NIST, and MMS with the data resulting from the analyses.
10. Expected date for completion of the analyses.

Costs incurred for providing samples (packaging, shipping, etc.) will be borne by the requester. In addition, samples have to be cryogenically homogenized and divided into aliquots (7-10 g) before being released for analysis. Only those samples being analyzed as part of the *AMMTAP*'s sample stability monitoring are routinely homogenized. Therefore, requests for tissue samples that have not been homogenized as part of these routine *Archive* procedures will incur an additional cost that will have to be borne by the individual or organization making the request.

The *AMMTAP* publishes Annual Tissue Inventory Reports. These reports provide the current inventory of tissues maintained in the *Archive* and the results of any chemical analyses.

Direct inquiries regarding sample access to:

Paul R. Becker
NOAA
222 West 8th Ave. #56
Anchorage, Alaska 99513
(907) 271-3032

PROTOCOL

Sample collections are conducted in cooperation with research and resource management programs of federal, state, and local agencies and organizations. Samples to be archived, including those obtained incidentally, are collected, documented, and handled in accordance with the standard protocols developed for the project in 1987, and which are consistent with those employed by the NBSB, NIST. Sample storage and inventory procedures are in accordance with those routinely performed at the NBSB. This includes specimen storage under liquid nitrogen vapor at -150°C.

The original collection protocols for this project were modifications of those used by the NBSB for the collection of human liver samples (Harrison *et al.*, 1981; Zeisler *et al.*, 1983a), and protocols used by NOAA's Status and Trends Program for the collection of fish, bivalves, and sediment samples for the purpose of specimen banking and environmental contaminant analysis (Lauenstein, 1986; Lauenstein and Young, 1986). This protocol is a revision of the original protocol (Becker *et al.*, 1988) and has deleted the muscle tissue sections, since muscle tissue is no longer collected for the NBSB.

The quality of logistic and support facilities vary within Alaska. The necessity for collecting materials in remote areas under relatively primitive conditions will probably require the development of more than one protocol. Within the limitations imposed by the conditions under which collections are made, the intent of each of the collection protocols is to obtain fresh, well-defined tissue samples uncontaminated by extraneous sources of trace elements and organic compounds, and to package and transport these samples as quickly as possible under conditions which eliminate or minimize chemical changes within the tissues prior to storage.

Materials

The following materials are used for collecting marine mammal tissue samples:

Container with blue ice for transporting bagged samples from collection site to processing site,
Teflon sheeting with adhesive backing for covering processing surfaces (usually lab benches),
Dry shippers (LN_2 cooled) with shipping container,
LN_2 in container for freezing samples on site,
Dewar and lid,
Lab coats (disposable),

Insulated gloves, safety glasses, and tongs for handling the LN_2 and frozen samples,
Balance for weighing samples (triple-beam balance is suitable),
Surgical scissors, forceps and screwcap vials with buffered formalin for histological samples,
Labels for exterior of sample jars,
Tape for securing exterior jar labels,
Shipping labels,
Data recording forms,
5′ x 10′ polyethylene sheet,
Duct tape
Waterproof pen for writing on duct tape, and
Garbage bags for collecting used materials in the field for later disposal.

In addition, the collection of two replicates of a single sample requires:

4 pairs non-talced vinyl gloves,
2 Teflon FEP (Fluorinated ethylene propylene) bags or sheets (14" X 16") for clean working surfaces,
1 Teflon FEP bag (12" X 12") for transporting sample from the field to the processing facilities,
2 Teflon jars (180 mL, 49 mm diameter, 120 mm length) with lid labels,
2 500-mL Teflon bottles containing high purity distilled or best available water for rinsing samples,
1 Titanium blade/Teflon TFE (Tetrafluoroethylene) handle knife, and
High grade ethanol for rinsing knife (1 L).

For the collection of associated biological/environmental data, the following equipment is also required:

Thermometer for air temperature,
Measuring tape (metric),
Heavy duty scales for weighing animal (where practical),
Short metric ruler for measuring blubber thickness, and
Camera for recording field procedures (optional).

The materials listed above for the tissue sampling are supplied by the NBSB to provide uniformity in the collection materials. The Teflon/titanium knives used in the protocol were fabricated at NIST specifically for the NBSB program and are cleaned before use according to the procedures described in the *Appendix, Procedures for Cleaning Titanium Knife*, page 32). The Teflon bags and sheets are obtained precleaned and specially packaged from the manufacturer (Clean Room Products, Ronkonkoma, New York). The Teflon jars are obtained from Savillex Corp (Minnetonka, Minnesota)

and are cleaned at the NBSB as described in the *Appendix, Procedure for Cleaning Teflon Jars and Implements*, page 31.

Field Kit

The items that must be transported to the field are provided in either two sturdy containers (one for samples and one for equipment and supplies) or in a single container (such as an ice chest) divided into two compartments, one for samples and one for equipment and supplies. Although the amount of materials in the field kit depends on the maximum number of animals to be sampled at any one time, the following is an itemization of what is required by two people for sampling one animal:

Blue ice in sample container,
Data recording forms,
2 Lab coats (disposable),
5′ X 10′ polyethylene sheet,
Package of non-talced vinyl gloves,
Teflon FEP bags packaged in Teflon,
Titanium blade/Teflon TFE handle knives packaged in Teflon,
Stainless steel knife,
Surgical scissors and forceps
500-mL Teflon bottle containing high purity distilled water,
500-mL Teflon bottle containing high grade ethanol,
Duct Tape for sealing Teflon sample bags,
Waterproof pen for writing on duct tape,
Thermometer for air temperature,
Measuring tape (metric),
Short metric ruler for measuring blubber thickness, and
Heavy duty scales for weighing animal (where practical).

Data Recording Forms: Instructions

Examples of the data recording forms are provided on pages 16-17. Standard biological/environmental information is recorded on the first page (page 16). The boxes at the top of this page provide space for the NBSB identification codes. These are assigned to the samples when they arrive at the *Archive*. Other information recorded on this page are:

1. *Individual ID Number*. This is a 10 digit alphanumeric code assigned by the *AMMTAP* before collections are made. The first three numbers (692) refer to the Research Unit number of this project. The next four letters are abbreviated common names for the species sampled (Table 2). The last three numbers are

ascending numbers that identify the individual animal sampled.

Table 2. Abbreviated common names to be used in the *Individual ID Number* of the data recording forms.

Common Name	Scientific Name	Abbreviation
Polar bear	*Ursus maritimus*	PLBR
Sea otter	*Enhydra lutris*	SEOT
Harbor seal	*Phoca vitulina*	HBSL
Spotted seal	*P. largha*	SPSL
Ringed seal	*P. hispida*	RGSL
Bearded seal	*Erignathus barbatus*	BDSL
Northern fur seal	*Callorhinus ursinus*	FRSL
Steller sea lion	*Eumetopias jubatus*	STSL
Pacific Walrus	*Odobenus rosmarus divergens*	WLRS
Bowhead whale	*Balaena mysticetus*	BWHD
Belukha whale	*Delphinapterus leucas*	BLKA
Dall's porpoise	*Phocoenoides dalli*	DLPR

2. *Species*. Genus/species name.

3. *Sample Source*. Information pertinent to identifying the agency, person(s), research, or management program providing or aiding in providing the samples is entered here.

4. *Site ID*. The most common name of the location where the animal is sampled (killed). This should be as specific as is practical.

5. *Lat. Long*. The latitude and longitude of the location where the animal is sampled. This should be to the nearest tenth of a minute, if possible.

6. *Time of Death*. Recorded as the day, month, year, and hour. The month should not be numbered, but should be written in abbreviated form (Jan, Feb, May, Apr, May, Jun, Jul, Aug, Sep, Oct, Nov, Dec) and the hour should be on a 24-hour basis (example, 6:00 pm is reported as 1800).

7. *Method of Collection*. The method by which the animal is sacrificed.

8. *Intermediate Storage*. Method of storage of animal carcass before removal of tissues. If tissues are taken immediately after death, no entry is necessary.

9. *Weather Conditions*. Space is provided for the notation of weather conditions occurring during sampling (wind, temperature, precipitation), particularly anything that would be pertinent relative to possible contamination sources.

10. *Sex, Age, and Method of Age Estimation*. Blocks are provided for indicating the sex of the animal and the age (in years). Method of age estimation and the name of the individual making that determination should also be recorded here.

11. *Weight*. Estimated or measured weight of the animal sampled should be recorded in the space provided.

12. *Length*. This is measured as snout to tail tip length along a straight line for pinnipeds, sea otters, and polar bears, and as snout to fluke notch length along a straight line for cetaceans.

13. *Axillary Girth*. This is the circumference of the animal measured just posterior of the front flippers.

14. *Fluke Width*. This is measured from tip to tip across the widest part of the fluke while the animal is lying flat, dorsal side up if possible.

15. *Blubber Thickness*. For pinnipeds and small cetaceans, such as the belukha, this is the sternal blubber thickness. For large cetaceans, such as the bowhead whale, the measurement might have to be made midway along the side depending on how the animal is laying.

16. *For Females*. Blocks are provided to note if the animal sampled is lactating or pregnant.

17. *General Appearance of Individual*. Any comments which describe the healthy or unhealthy appearance of the animal, external parasites, evidence of trauma, or body condition of the animal.

18. *General Appearance of Organs*. Any unusual appearances of any of the internal organs, particularly those to be sampled. If they all appear normal, note this also.

19. *Stomach Contents*. Food items if present are identified and some impression of degree of fullness recorded. If internal parasites are present, they are also noted.

20. *Additional Samples*. Samples collected from the same animal for other research purposes are recorded in this space. This information includes the kind of sample(s), for what purpose, and the name and location of the individual receiving the samples.

The second page of the data recording form (page 17) provides for the entry of data and information specific to the tissue samples, themselves.

21. *Individual ID Number.* This is the same number as found on the first page (Item #1). It is entered here in case the two pages are accidentally separated.

22. *Sample Type.* The type(s) of tissue samples collected from the individual animal are indicated by circling the appropriate category or by writing the name in the space provided.

23. *Time of Collection.* Entered the same way as in Item #6 on the first page of the data recording form. This is the time at which the tissues are removed from the animal in the field.

24. *Collected by.* Name of the individual actually removing tissue samples from the animal in the field.

25. *Intermediate Storage.* Space is provided to indicate how tissue samples collected in the field are stored during transport to the processing site (method of cooling, transport containers, etc.).

26. *Time of Preparation.* Entered the same way as in Item #6 on the first page of the data recording form. This is the beginning time at which tissues are processed into subsamples and placed in containers in preparation for LN_2 freezing.

27. *Time of LN2 Freezing.* Entered the same way as in Item #6 on the first page of the data recording form. This is the time at which the samples are frozen in LN_2. It is basically concurrent with the time at which preparation of the subsamples are completed.

28. *Time Shipped From Site.* Entered the same way as in Item #6 on the first page of the data recording form. This is the time at which the samples leave the sample processing site enroute to the *NBSB*, Gaithersburg, Maryland.

29. *Time Received at Archive.* Entered the same way as in Item #6 on the first page of the data recording form. This is the time at which the samples are received at the NBSB, Gaithersburg, Maryland.

30. *Processor.* The name of the individual(s) processing the samples for LN_2 freezing and shipment to the NBSB.

31. *Shipper*. The name of the person responsible for shipping the samples to the NBSB.

32. *Receiver*. The name of the individual receiving the samples at the *NBSB*.

33. *Protocol*. If the standard or a modified protocol was used to collect or process the samples, this is noted here. If a modified protocol, the nature of the modification is recorded.

34. *Remarks*. Space is provided for any additional remarks pertinent to the collection, processing, or archiving of the tissue samples.

35. *Histological Samples*. If samples are collected for histological slides, these are recorded here. In all cases, histological samples are to be taken from B subsamples only. The *Individual/Organization* making the slides, the *Final Destination* of the slides, the kind of *Tissues Sampled*, and the method of sample preservation (if appropriate) are recorded here.

36. *Sample Weight*. The weights of subsamples are recorded here. Note, that in some cases the collection of whole organs might be necessary in order to provide enough tissue for a subsample. If that is the case, and if these tissues are paired, the location of the specific organ should be noted by the subsample space (In the example, the entire right kidney is subsample A and the left kidney is subsample B).

37. *Prepared by*. The name of the individual filling out the data recording form.

All of the appropriate information for express shipping the samples are provided in the lower right hand corner of the second page. This includes the street, building, and room address of the *NBSB*, as well as the name and telephone number of the individual responsible for receiving the samples.

NATIONAL BIOMONITORING SPECIMEN BANK

Sampling Data - NOAA/MMS

Alaska Marine Mammal Tissue Archival Project

Individual ID Number _____ Species _____

Sample Source _____

Site ID _____ Lat. _____ Long. _____

Time of Death: [ ][ ][ ] [ ] Method of Collection _____

day mo yr hr

Intermediate Storage (temp/remarks) _____

_____

Weather Conditions (wind, temp., precip.) _____

_____

Sex: ☐ Male ☐ Female Age _____ Method of Age Estimation _____

Weight: Estimated _____ Weighed _____ kg.

Length: Snout-Tail Tip _____ cm.

Snout-Fluke Notch _____ cm.

Axillary Girth _____ cm.

Fluke Width _____ cm.

Blubber Thickness _____ cm.

For Females:

☐ Lactating

☐ Pregnant

General Appearance of Individual:

General Appearance of Organs:

Stomach Contents:

Additional Samples:

Individual ID Number _____

Sample Type: ☐ Liver ☐ Kidney ☐ Blubber Other _____

Time of Collection: day mo yr hr Collected by _____

Intermediate Storage (temp/remarks) _____

_____

Time of Preparation: day mo yr hr Processor _____

Time of LN2 Freezing

Time Shipped From Site: Shipper _____

Time Received at Archive: Receiver _____

Protocol: ☐ Standard ☐ Modified (Please note modification below)

Remarks:

Histological Samples:

Individual/Organization _____

Final Destination _____

Tissues Sampled _____

Sample Weight:	A	B		A	B
Liver	g.	g.	Blubber	g.	g.
Kidney	g.	g.	Other	g.	g.

Prepared by: _____
Name (print)

_____
Signature

A copy of this form should be shipped with samples to:

National Institute of Standards and Technology
Bldg 235, Rm B118
Gaithersburg, MD 20899

Attn: Barbara Koster
(301) 975-6291

Sampling Procedure

The collection protocol consists of three stages: tissue removal from the individual animal, tissue processing to obtain representative samples, and packaging/shipping of the samples to the *Archive* (Figure 2). The division of the protocol into stages is used as an aid in organizing and simplifying the collection procedures.

Stage I, tissue removal, will occu indoors or out-of-doors in the field under conditions of limited control. Procedures for tissue removal will vary depending upon the group of mammals being sampled (e.g., pinnipeds vs cetaceans), but should be the same for individuals of the same species.

Stage II, tissue processing, will occur indoors in the majority of cases and under laboratory conditions where possible; this includes shipboard laboratories.

Stage III, sample packaging and shippin, should be relatively standard for all tissues and should not vary, while the processing of tissues might vary depending on the availability of laboratory facilities near the collection site and the tissue type to be sampled.

Stage I, Tissue Removal

1. The size of the tissue sample removed from an animal should be sufficient to provide two subsamples of 150-200 g, each, for archival. This amount of material is required in order to maintain long-term archived samples plus aliquots for periodic analysis.

2. The anatomical location of tissue removal is specified in order to maintain consistency and comparability between the same tissue types.

3. Sterile, cleaned, non-talced vinyl gloves are used by all personnel involved in sample removal and handling. Precaution must be taken throughout the procedures to reduce the risk of any contamination that might alter the sample. Contamination may originate from the individual performing the work (do not smoke during the procedure), the atmosphere, the skin of the animal, the instruments used in the dissection, and any chemicals that happen to be in the area where the work is being performed. (Refer to *Appendix, Contamination Sources*, page 31)

4. No animal is considered a candidate for sampling if tissues cannot be collected within 6 hours after death

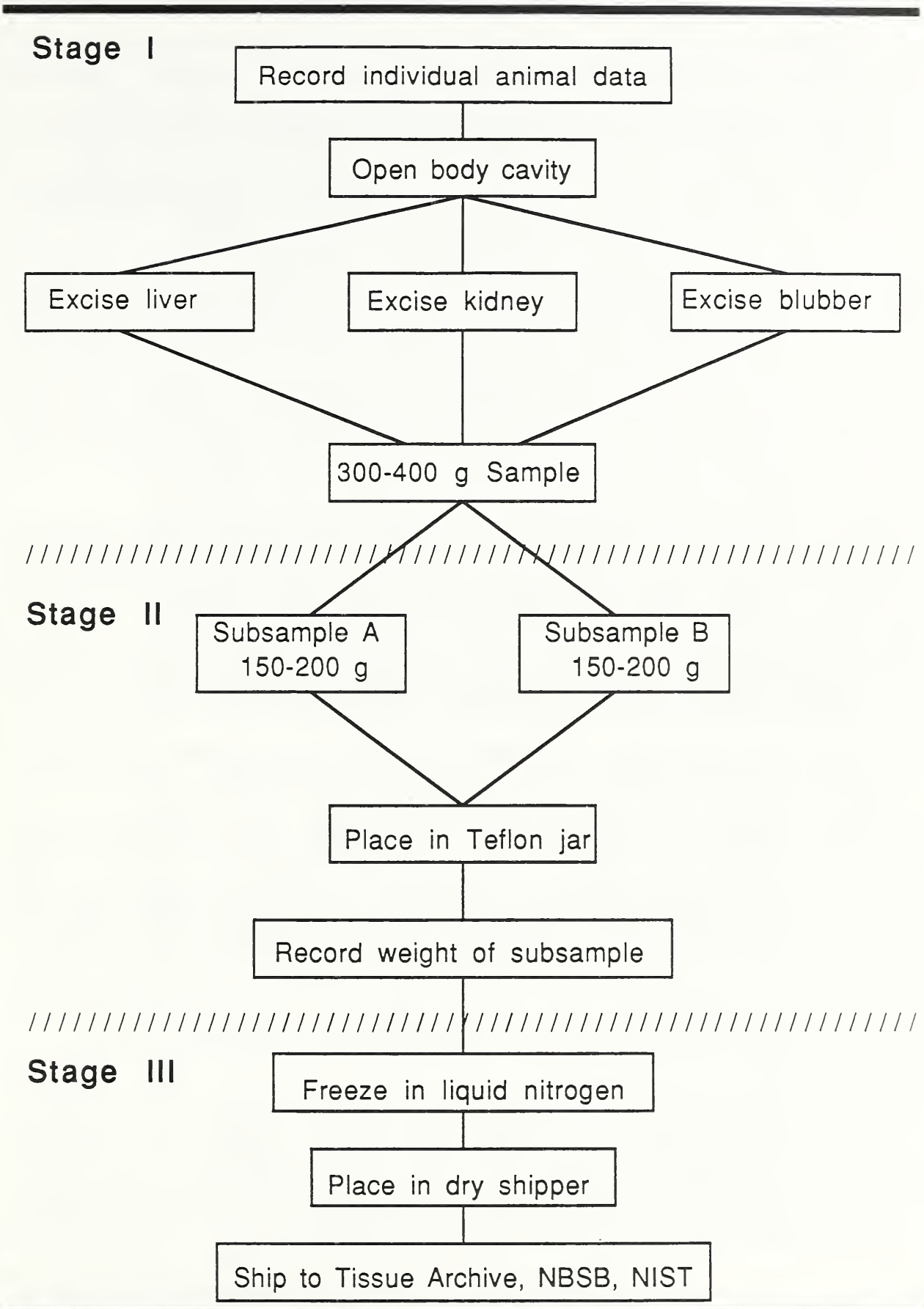

Figure 2. Generalized collection protocol.

and if handling of the carcass between time of death and tissue removal can not be documented.

5. *Procedures for Pinnipeds and Sea Otters* (from Fay *et al.*, 1979):

   a. Record the weather information and individual animal data on the data recording form.

   b. For carcasses that can be moved, place carcass ventral side up on 5′ x 10′ polyethylene sheet; straighten the spine (e.g., by grasping the head and pulling). For animals that can not be moved, place the polyethylene sheet on the left side of the carcass to provide a working surface beside the animal. Measure the length (tip of snout to tip of tail flesh, in a straight line) and axillary girth (cm).

   c. Make an incision 4-5 cm long over the sternum, midway between the axillae, cutting through the skin and blubber. Measure the blubber thickness (mm), not including skin, at this point.

6. *Procedures for Cetaceans* (modified from Fay *et al.*, 1979; procedures are consistent with those used in the North Slope Borough Belukha Harvest Survey):

   a. Record the weather information and individual animal data on the data recording form.

   b. For animals that can be moved, place carcass left side up on 5′ x 10′ polyethylene sheet. For animals that can not be moved, place the polyethylene sheet to the left side of the carcass to provide a working surface beside the animal. Measure the length (tip of snout to fork of tail, in a straight line), fluke width, and axillary girth (cm). In some cases, the size of the animal may not allow for measuring the axillary girth.

   c. Make an incision over the sternum, midway between the axillae, cutting through the skin and blubber. The incision should be small enough to prevent distortion, but its size will depend on the size of the animal and thickness of its blubber. Measure the blubber thickness (mm) at this point. Blubber thickness includes the skin.

      If it is impossible to make this measurement over the sternum, make an incision through the skin and blubber about midway along the side and measure the blubber thickness (mm) there. Make note of this on the data recording form.

7. The tissue samples are removed as soon as possible after opening the body cavity. Opening of the body cavity and initial cutting of the skin to expose muscle and adipose tissue may be performed with high quality stainless steel dissection tools previously rinsed in the high purity water.

   a. *Procedures for Pinnipeds and Sea Otters* (from Fay *et al.*, 1979): Remove the ventral body wall from chin to anus, cutting through the costal cartilages, and lay it out, skin side down, to one side as a work area. Confirm the gender.

      Note! In the case of the northern fur seals, the pelt is traditionally removed from the animal before the body cavity is opened. For this species, the animal is placed on its back on the polyethylene sheet and incisions are made around the neck and flippers, and through the skin from chin to anus. The animal is then turned over on its ventral side (still on the polyethylene sheet), the head is held in place by a forked steel bar, and the skin of the back of the head is grasped and pulled to the posterior to remove the entire pelt. The body cavity is then opened and the internal organs removed as described below.

   b. *Procedures for Cetaceans* (modified from Fay *et al.*, 1979): With the animal lying on its side, remove the skin and blubber from the left lateral body wall and lay it out, skin side down, to one side as a work area. Confirm the gender.

   c. Adipose Tissue (Blubber). The anatomical site of blubber removal will depend on the distribution of fat layers on the animal and will, therefore, be rather species specific.

      (1) *For animals with relatively thin blubber layers, such as fur seals*: Using the titanium knife, remove sections of blubber from along the backbone until the required 400 g are obtained. Any small portions of muscle tissue will be removed at the processing site. Avoid collecting blubber from near the areas where the initial cuts through the skin were made since these areas are contaminated with loose hair.

      (2) *For animals with relatively thick blubber layers*: In the region of the sternum (or to the left of the point where the blubber thickness measurement was made), remove a

rectangular section (400 g) of blubber using the titanium knife previously rinsed in high purity water. This section should be a vertical section from just below the skin to the surface of the muscle. This sample excision is made by separating the layer of blubber to the left of the ventral mid-line from the overlying skin and then from the underlying muscle layer using the titanium knife. Continuing to use the titanium knife, the rectangular section of separated blubber is then removed from the animal. Place the blubber sample in a clean Teflon bag for immediate transport to the processing area.

d. Liver. Note the general appearance of the liver before removal, including any unusual color, texture, shape, etc.

(1) *For the smaller species (northern fur, ringed, spotted, and harbor seals, and sea otters)*: Remove the entire liver from the animal and place in a clean Teflon bag for immediate transport to the processing area. Ligaments of the liver may be cut using surgical scissors which have been previously rinsed. (If liver membrane is ruptured, the sample is not acceptable).

(2) *For the larger species (walrus, bearded seals, sea lions, and cetaceans)*: Using the titanium knife, remove a 300-400 g section from the liver and place in a clean Teflon bag for immediate transport to the processing area. For animals with multi-lobed livers (pinnipeds), this section is taken from the posterior portion of the left anterior lobe. For animals with liver divided into two lobes by a shallow indentation in the posterior edge (cetaceans), this section is taken from the posterior portion of the left lobe. (If liver membrane is ruptured, the sample is not acceptable).

e. Kidney. Note the general appearance of the kidney before removal, including any unusual color, texture, shape, etc.

(1) *For the smaller species (northern fur, ringed, spotted, and harbor seals, and sea otters)*: Remove both kidneys from the animal and place in a clean Teflon bag for immediate transport to the processing area. Both kidneys are required in order to provide a

total sample size of 300 g. (Note which is the left and which is the right kidney). Attachments of the kidney may be cut using surgical scissors which have been previously rinsed. (If the kidney membrane is ruptured, the sample is not acceptable).

(2) *For the larger species (walrus, bearded seals, sea lions, and cetaceans)*: Remove the left kidney from the animal and place in a clean Teflon bag for immediate transport to the processing area. Attachments of the kidney may be cut using surgical scissors which have been previously rinsed. (If the kidney membrane is ruptured, the sample is not acceptable).

If the entire kidney is too large to be transported back to the processing area, remove a 300-400 g. section from the posterior end of the left kidney using the titanium knife and place in a clean Teflon Bay for transport

8. For the pinnipeds and toothed cetaceans, the growth layers of the teeth are used to determine age. The procedures for extracting and preparing the teeth for examination are given in the *Appendix*, page 33.

Stage II, Tissue Processing

1. Tissue processing takes place indoors within a laboratory facility and under a clean air hood, when possible. At a minimum, the tissue processing occurs in a covered and enclosed area free of obvious sources of contamination, such as cigarette smoke, fuel oil fumes and smoke, laboratory formaldehyde, etc. The processing area of the laboratory is cleaned to remove dust and the working surfaces covered with Teflon sheeting.

2. Only titanium knives are used to cut samples during Stage II. Two knife cleaning procedures are presented in the *Appendix*, page 32. *Cleaning Procedure I* can be performed at the processing site. *Procedure II* is performed only at the NBSB facilities, or other suitable laboratory.

3. If tissue sections are taken for histological work, this information is recorded in the appropriate space on the data reporting form. The results of the histological analysis should be reported to the NBSB. Any change in the physical location of the histological

slides should be reported to the NBSB. Investigators who intend to dispose of histological slides after analysis are encouraged to transfer these materials to the NBSB, where they will be cataloged, cross-referenced to archived tissue samples, and maintained in the NIST archives.

4. Liver

a. Remove the sample from the Teflon bag and, holding it over a sink, rinse the surface of the specimen with water from the Teflon bottle. Pour approximately 100 mL or more of the water from the Teflon bottle over the specimen to wash off blood and other fluid. Rub the specimen with gloved hand, if necessary, to remove blood and fluid. Allow the specimen to drain for several minutes. Place the rinsed specimen on a clean Teflon sheet.

b. The seal liver consists of four to eight long finger-like lobes. If the whole liver is transported to the processing facility, remove the two anterior lobes with the titanium knife to give two samples (Sample A and Sample B) of 150-200 g each. Each sample must fit in a Teflon jar with a volume of 180 mL (49 mm diameter and 120 mm length).

c. If the liver sample is 300-400 g, using the titanium knife divide the specimen into two equal samples (Sample A and Sample B) of 150-200 g each. Each sample must fit in a Teflon jar with a volume of 180 mL (49 mm diameter and 120 mm length).

d. Tare the weight of the pre-cleaned Teflon jar, place the individual sample in the tared Teflon jar, and weigh each individual sample. Record the weights on the data recording form and sample labels. Affix the sample labels to the jars with wide tape and place the lid labels in the recessed slot on the jar lids.

e. Rinse the titanium knife with the water and rub with the gloved fingers to remove all blood and fluids from the knife before they have time to become dried on. Rinse the knife with ethanol and air dry.

f. Continue to *Stage III, Tissue Freezing and Shipping*.

5. Kidney

a. Remove the specimens from the Teflon bag; rinse the surface of each kidney with water from the Teflon bottle. Pour approximately 100 mL or more of the water from the Teflon bottle over each kidney to wash off blood and other fluid. Rub each specimen with gloved hand, if necessary, to remove blood and fluid. Allow the kidneys to drain for several minutes.

b. In some cases each kidney will approach the weight of the required specimen size for each subsample (150 g) and no subsampling will be necessary. The right kidney will provide Sample A and the left kidney will provide Sample B. If subsampling is required (or if cutting of the sample is necessary to fit the sample into the jar), use the titanium knife to remove a 150 g section from the kidney. Each sample must fit in a Teflon jar with a volume of 180 mL (49 mm diameter and 120 mm length).

c. If the left whole kidney was collected from the animal or if samples of kidney tissue were collected, remove two subsamples of 150 g each from the kidney sample using the titanium knife. Each sample must fit in a Teflon jar with a volume of 180 mL (49 mm diameter and 120 mm length).

d. Tare the weight of the pre-cleaned Teflon jar, place the individual sample in the tared Teflon jar, and weigh each individual sample. Record the weights on the data recording form and sample labels. Affix the sample labels to the jars with wide tape and place the lid labels in the recessed slot on the jar lids.

e. Rinse the titanium knife with the water and rub with the gloved fingers to remove all blood and fluids from the knife before they have time to become dried on. Rinse the knife with ethanol and air dry.

f. Continue to *Stage III, Tissue Freezing and Shipping*.

6. Blubber

a. Remove the specimen from the Teflon bag. Pour approximately 100 mL or more of the water from the Teflon bottle over the specimen to wash off blood and other fluid. Allow the specimen to drain for several minutes. If the sample retains the

original edge produced during the opening of the body cavity, this should be trimmed away using the titanium knife.

b. Use the titanium knife to remove any remaining portions of muscle attached to the blubber.

c. Using a clean titanium knife, divide the specimen into two equal samples (Sample A and Sample B) of 150-200 g, each. Each sample must fit in a Teflon jar with a volume of 180 mL (49 mm diameter and 120 mm length).

d. Tare the weight of the pre-cleaned Teflon jar, place the individual sample in the tared Teflon jar, and weigh each individual sample. Record the weights on the data recording form and sample labels. Affix the sample labels to the jars with wide tape and place the lid labels in the recessed slot on the jar lids.

e. Rinse the titanium knife with the water and rub with gloved fingers to remove all blood and fluids from the knife before they have time to become dried on. Rinse the knife with ethanol and air dry.

f. Continue to *Stage III, Tissue Freezing and Shipping*.

Stage III, Tissue Freezing and Shipping

1. Freeze each sample by immersing in the LN_2 for 10 minutes.

   Liquid nitrogen should not be stored in sealed containers. Personnel handling LN_2 are cautioned to wear boots, cuffless trousers, non-absorbent aprons, loose insulating gloves, and safety glasses.

2. The LN_2 shipper should be filled with liquid nitrogen for at least 6 hours to fully prepare it for shipping. This is required to fully saturate the absorbent inside the shipper. Pour off the excess LN_2 and place the frozen samples in the shipper (10-12 sample boxes per shipper).

3. Once full, transport the shippers to the NBSB, NIST; do not store in intermediate freezers.

4. Double check the data recording forms for completeness and accuracy. Any deviations or modifications of the protocol must be noted on each form.

5. Place a copy of the completed forms in the shipper; another copy is retained by the collector for project records.

6. Ship samples within 48 hours or as soon as possible after sample collection using 24 hour express package service to:

National Institute of Standards and Technology
Building 235, Room B-125
Gaithersburg, Maryland 20899
Attn: Barbara Koster (301) 975-6291

In most cases, samples are shipped commercial-air to Anchorage and then 24 hour express package service to the NBSB *Archive*. The shippers must not contain LN_2 when shipped. Maximum holding time for the shippers is 10-12 days. Shipments are not made late in the week, i.e., Thursday, or Friday, or before holidays, unless special arrangements have been made with the shipping service and NIST.

7. The Specimen Bank personnel are notified by telephone as soon as possible after the specimens are shipped:

Barbara Koster	(301) 975-6291 (FTS, 879-6291), or
Steve Wise	(301) 975-3112 (FTS, 879-3112)

Sample Archival

Samples are received at NIST and transported to the NBSB facility. The shippers are unpacked and samples are inspected for any packaging problems and for unsuitable temperatures. Data recording forms and samples are compared to insure that they correspond and that all information has been included. These samples are stored in a temporary liquid nitrogen (LN_2) freezer and are logged into the temporary storage log book. They remain in temporary storage until assigned an *NBSB* number and permanent LN_2 freezer space.

When the samples are moved into the permanent freezer location, a storage form, which contains storage location information, is completed, and the information is entered into the inventory form on the NBSB personal computer. The samples are placed in cylindrical cardboard tubes (6.0 cm diameter x 63.5 cm length); each tube will hold up to five samples. These samples will remain stored in the LN_2 freezer at about -150°C until they are requested for analysis.

The duplicate samples of each tissue (Samples A and B) collected for the *AMMTAP* are stored in different LN_2 freezers to provide additional security. Sample "A" is intended for long-term storage while sample "B" is available for any analyses as required (see below). Samples to be analyzed are homogenized using a cryogenic grinding procedure designed to reduce the likelihood of changes in sample composition due to thawing and refreezing (Zeisler *et al.*, 1983b). The sample homogenate is divided into aliquots of approximately 10 15-mL Teflon jars (7-10 g homogenate/jar) and 2-3 90-mL Teflon jars (30 g/jar). Aliquots that are not required for immediate analysis are returned to the LN_2 freezer for storage.

Analysis of Specimens

As part of the *AMMTAP*, NIST analyzes 10-15% of the specimens to determine the concentrations of selected organic and inorganic constituents. These analyses provide baseline data for the following purposes:

1. for evaluating the stability of the specimens during long-term storage,

2. to provide some real-time measure of contaminant concentrations for monitoring purposes,

3. to provide a baseline for comparing with results from samples collected in the future in order to monitor long-term trends in pollution, and

4. for comparing with data obtained by other laboratories on subsamples from the *AMMTAP*, or similar samples collected at the same time from the same sites (i.e., quality assurance).

Analytical results are reported in Annual Reports of the *AMMTAP*.

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APPENDIX

PROCEDURE FOR CLEANING TEFLON JARS AND IMPLEMENTS

All of the implements and containers that contact the specimens in the National Biomonitoring Specimen Bank (NBSB) are thoroughly cleaned before each use to remove possible organic or inorganic trace contaminants. The Teflon sheets and bags used for the NBSB are precleaned and specially packaged by the supplier (Clean Room Products, Ronkonkoma, New York). All other Teflon items (e.g., cryogenic grinding mills, jars, scoops, and spatulas) are cleaned at NIST using the procedure described below. This cleaning procedure is performed at the NBSB facility in its class 100 clean room in polyethylene vats contained in a Teflon lined hood. The chemicals are removed from the vats using a Teflon lined pump. In addition to the normal clean room clothing (lab coats, booties, and hoods), safety goggles and rubber gloves are required for handling the solvents and acids. The cleaning procedure is as follows:

Chloroform	1 hour*
Ethyl Alcohol, 99.5%	1 hour*
HPLC water rinse (pour over equipment and suction off immediately)*	
Hydrochloric Acid, 1:2 dilution	4 hours*
HPLC water rinse*	
Nitric Acid, 1:2 dilution	4 hours*
HPLC water rinse*	
Ethyl Alcohol, 99.5 % (new bottle) rinse	
HPLC water rinse (new bottle)	
HPLC water rinse (new bottle)	

Note: cover the implements completely with each specific chemical.

**These items may be used a maximum of four times before discarding, unless there is obvious contamination.*

PROCEDURES FOR CLEANING TITANIUM KNIFE

There are two cleaning procedures for the titanium knives and other reusable implements: the first is to be completed after finishing a sample and the second is used after knife sharpening or after excessive contamination. The titanium knives should be sharpened only with the silicon carbide stone provided and only these knives should be sharpened with this stone to avoid cross contamination.

Cleaning Procedure I

After placing tissue sections in bags and before leaving the sample preparation area, the knife should be rinsed using high-purity water. While rinsing, and with gloved hands, run fingers over the blade and handle of the knife to help remove any adhering blood or tissue. This is best done before any fluid or tissue has a chance to dry on the knife. In the laboratory, the knife should be rinsed again, as above, with water and then with ethanol. the knife is then placed on a clean surface (do not touch the blade) and allowed to air dry, preferably in a laminar flow hood. The knife should then be place in a Teflon bag, made from the Teflon sheets, for storage and transported to the next sampling site. The implements should at no time be touched with ungloved hands.

Cleaning Procedure II

This procedure should be applied after excessive contamination of the implements and always after a knife is sharpened. Rinse the implement as described in cleaning procedure I. The knife may be disassembled to clean if necessary. In the laboratory, the implement is placed in a clean container and covered with 99.5% ethyl alcohol for one to two hours. The implement is then covered with high purity water overnight. The implement is covered with dilute hydrochloric acid (one part hydrochloric acid and ten parts high-purity water) for half an hour. The implement is then removed from the acid, rinsed with high-purity water, and covered with dilute nitric acid (one part nitric acid and ten parts high-purity water) for half an hour. The implement is again removed from the acid and rinsed with high-purity water. The implement is removed from the washing container and placed on a clean surface to air dry, preferably in a laminar flow hood. Only the knife handle should touch the drying surface. The clean, dry implement should be stored in Teflon bags made from Teflon sheets.

PROCEDURES FOR EXTRACTING AND PREPARING MARINE MAMMAL TEETH FOR AGE DETERMINATION

For odontocetes, remove the mandibles containing the teeth by first removing the skin and muscle from the outside of the bone with a knife, then cutting through the mandibles posterior to the rear-most teeth with a hatchet or saw. Sever the attachment of the mandibles to the tongue and remove them from the animal. The teeth can be removed later for preparation and age determination.

For the pinnipeds, either upper or lower canine teeth are used to determine age. If the upper canine teeth are collected, use a bone saw, bolt cutter, or other appropriate tool to remove the upper maxilla containing the canines. The lower canines can be collected by first removing the skin from the lower jaw with a knife, severing the masseter muscle lying over the zygomatic bone, then inserting the knife into the zygomatic arch to sever the muscles attaching the mandible to the skull. The entire mandible containing the lower canines can then be retained for later tooth removal and preparation.

To prepare the teeth, boil the maxilla or the mandible containing the teeth for 20 minutes to soften the bone. Remove the teeth and extract the pulp from each. Boil the teeth for 10-15 minutes in a solution of one tablespoon sodium triphosphate per one liter of water. Dry and label each tooth with the following information:

AMMTAP ID Number
Species
Sex
Collection Date

NIST-114A
(REV. 3-89)

U.S. DEPARTMENT OF COMMERCE
NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY

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11. ABSTRACT (A 200-WORD OR LESS FACTUAL SUMMARY OF MOST SIGNIFICANT INFORMATION. IF DOCUMENT INCLUDES A SIGNIFICANT BIBLIOGRAPHY OR LITERATURE SURVEY, MENTION IT HERE.)

In 1987, the Alaska Marine Mammal Tissue Archival Project (AMMTAP) was established to archive a representative collection of Alaska marine mammal tissues for future analyses and documentation of long-term trends in environmental quality. The original field collection protocols for the AMMTAP were developed based on a 1987 pilot study of northern fur seal sampling. Since 1987, the sample collection protocol has been continually evaluated and updated to include the experience gained from sampling additional species under different conditions in Alaska. This report contains the revised protocol for the collection of marine mammal tissues for long-term storage as part of the AMMTAP.

12. KEY WORDS (6 TO 12 ENTRIES; ALPHABETICAL ORDER; CAPITALIZE ONLY PROPER NAMES; AND SEPARATE KEY WORDS BY SEMICOLONS)

marine mammals, Alaska, arctic research, sample collection, specimen banking, long-term storage, subsistence hunting

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